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## Antimetastatic potential of amide-linked local anesthetics: inhibition of lung adenocarcinoma cell migration and inflammatory Src signaling independent of sodium channel blockade

Piegeler, Tobias ; Votta-Velis, E Gina ; Liu, Guoquan ; Place, Aaron T ; Schwartz, David E ; Beck-Schimmer, Beatrice ; Minshall, Richard D ; Borgeat, Alain

**Abstract:** **BACKGROUND:** Retrospective analysis of patients undergoing cancer surgery suggests the use of regional anesthesia may reduce cancer recurrence and improve survival. Amide-linked local anesthetics have antiinflammatory properties, although the mechanism of action in this regard is unclear. As inflammatory processes involving Src tyrosine protein kinase and intercellular adhesion molecule-1 are important in tumor growth and metastasis, we hypothesized that amide-linked local anesthetics may inhibit inflammatory Src-signaling involved in migration of adenocarcinoma cells. **METHODS:** NCI-H838 lung cancer cells were incubated with tumor necrosis factor- in absence/presence of ropivacaine, lidocaine, or chloroprocaine (1 nM-100 M). Cell migration and total cell lysate Src-activation and intercellular adhesion molecule-1 phosphorylation were assessed. The role of voltage-gated sodium-channels in the mechanism of local anesthetic effects was also evaluated. **RESULTS:** Ropivacaine treatment (100 M) of H838 cells for 20 min decreased basal Src activity by 62% ( $P=0.003$ ), and both ropivacaine and lidocaine coadministered with tumor necrosis factor- statistically significantly decreased Src-activation and intercellular adhesion molecule-1 phosphorylation, whereas chloroprocaine had no such effect. Migration of these cells at 4 h was inhibited by 26% ( $P=0.005$ ) in presence of 1 M ropivacaine and 21% by 1 M lidocaine ( $P=0.004$ ). These effects of ropivacaine and lidocaine were independent of voltage-gated sodium-channel inhibition. **CONCLUSIONS:** This study indicates that amide-, but not ester-linked, local anesthetics may provide beneficial antimetastatic effects. The observed inhibition of NCI-H838 cell migration by lidocaine and ropivacaine was associated with the inhibition of tumor necrosis factor--induced Src-activation and intercellular adhesion molecule-1 phosphorylation, providing the first evidence of a molecular mechanism that appears to be independent of their known role as sodium-channel blockers.

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# Antimetastatic Potential of Amide-linked Local Anesthetics

## *Inhibition of Lung Adenocarcinoma Cell Migration and Inflammatory Src Signaling Independent of Sodium Channel Blockade*

Tobias Piegeler, M.D.,\* E. Gina Votta-Velis, M.D., Ph.D.,† Guoquan Liu, Ph.D.,‡ Aaron T. Place, Ph.D.,§ David E. Schwartz, M.D.,|| Beatrice Beck-Schimmer, M.D.,# Richard D. Minshall, Ph.D.,\*\* Alain Borgeat, M.D.††

### ABSTRACT

**Background:** Retrospective analysis of patients undergoing cancer surgery suggests the use of regional anesthesia may reduce cancer recurrence and improve survival. Amide-linked local anesthetics have antiinflammatory properties, although the mechanism of action in this regard is unclear. As inflammatory processes involving Src tyrosine protein kinase and intercellular adhesion molecule-1 are important in tumor growth and metastasis, we hypothesized that amide-linked local anesthetics may inhibit inflammatory Src-signaling involved in migration of adenocarcinoma cells.

**Methods:** NCI-H838 lung cancer cells were incubated with tumor necrosis factor- $\alpha$  in absence/presence of ropivacaine, lidocaine, or chloroprocaine (1 nM–100  $\mu$ M). Cell migration and total cell lysate Src-activation and intercellular ad-

### What We Already Know about This Topic

- Regional anesthesia is associated in some clinical studies with reduced risk of metastasis or late mortality in patients undergoing cancer surgery
- Local anesthetics have antiinflammatory effects that may participate in a reduced risk of metastasis, but their mechanisms are unknown

### What This Article Tells Us That Is New

- In cellular studies *in vitro* with lung cancer cells, amide- but not ester-local anesthetics reduced tumor cell migration and reduced signaling pathways important to tumor growth and metastases
- Regional anesthesia might reduce cancer metastasis by direct effects of absorbed amide local anesthetics on tumor cells

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hesion molecule-1 phosphorylation were assessed. The role of voltage-gated sodium-channels in the mechanism of local anesthetic effects was also evaluated.

**Results:** Ropivacaine treatment (100  $\mu$ M) of H838 cells for 20 min decreased basal Src activity by 62% ( $P = 0.003$ ), and both ropivacaine and lidocaine coadministered with tumor necrosis factor- $\alpha$  statistically significantly decreased Src-activation and intercellular adhesion molecule-1 phosphorylation, whereas chloroprocaine had no such effect. Migration of these cells at 4 h was inhibited by 26% ( $P = 0.005$ ) in presence of 1  $\mu$ M ropivacaine and 21% by 1  $\mu$ M lidocaine ( $P = 0.004$ ). These effects of ropivacaine and lidocaine were independent of voltage-gated sodium-channel inhibition.

**Conclusions:** This study indicates that amide-, but not ester-linked, local anesthetics may provide beneficial antimetastatic effects. The observed inhibition of NCI-H838 cell migration by lidocaine and ropivacaine was associated with the inhibition of tumor necrosis factor- $\alpha$ -induced Src-activation and intercellular adhesion molecule-1 phosphorylation, providing the first evidence of a molecular mechanism that appears to be independent of their known role as sodium-channel blockers.

**L**UNG cancer is the leading cause of death from cancer in males and the second leading cause of death in females<sup>1</sup>; effective treatment remains a challenge. Even if patients undergo complete tumor resection, including lymphadenectomy, there is still a high rate of relapse because of undetected micrometastasis.<sup>2–4</sup> Regional anesthesia with local anesthetics in combination with general anesthesia is commonly used in patients undergoing tumor resection of the lung.<sup>5</sup>

It is well known that amide-linked local anesthetics – as opposed to ester-linked compounds – can exert inhibitory effects on inflammatory processes,<sup>6</sup> *e.g.*, as ropivacaine has been shown to possess antiinflammatory properties in a model of pulmonary inflammation.<sup>7</sup> Also, the systemic use of lidocaine in patients undergoing colo-rectal surgery led to a decrease in inflammatory cytokine release as well as a shortened hospital stay.<sup>8</sup>

There is increasing evidence that inflammatory mechanisms may play an important role in the development and growth of cancer metastasis.<sup>9</sup> Cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) increase the expression of intercellular adhesion molecule-1 (ICAM-1), a cell surface receptor required for leukocyte adhesion<sup>10</sup> and tumor invasion,<sup>11</sup> in the H838 nonsmall cell lung cancer cell line.<sup>12</sup> ICAM-1 also facilitates tumor cell extravasation *via* the binding of neutrophils.<sup>13</sup> TNF- $\alpha$  is known to induce activation of Src protein tyrosine kinase, which functions as a regulator of endothelial permeability.<sup>14</sup> Src-mediated phosphorylation of ICAM-1 is necessary for neutrophil adhesion to the endothelium during acute lung inflammation and injury,<sup>15</sup> a process considered to induce vascular hyperpermeability.<sup>10</sup> In addition, Src is involved in signaling epithelial-to-mesenchymal transformation and extravasation of cancer cells,<sup>16,17</sup> processes that are necessary for solid tumor metastasis.<sup>18</sup>

Recently published retrospective analysis of patients undergoing cancer surgery showed a possible benefit of the perioperative use of regional anesthesia. Long-term survival after colon cancer surgery<sup>19</sup> and cancer recurrence after radical prostatectomy for prostate cancer<sup>20</sup> or breast cancer<sup>21</sup> were significantly improved by epidural anesthesia/analgesia compared with patient-controlled analgesia with opioids. The mechanism by which regional anesthesia might prove to be beneficial at the molecular level in this context is not yet known. We hypothesized that this effect might be related to the antiinflammatory properties of local anesthetics.

The primary goal of this study was to determine whether amide-linked local anesthetics (ropivacaine and lidocaine) attenuate TNF- $\alpha$ -induced Src activation and ICAM-1 phosphorylation in H838 human lung cancer cells and whether these effects are because of the inhibition of voltage-gated sodium channels (VGSC). Second, we examined whether lidocaine, ropivacaine, or chloroprocaine inhibit migration of this cancer cell line *in vitro*.

## Materials and Methods

### Cell Culture

The human NCI-H838 lung cancer cell line (CRL-5844, ATCC, Rockville, MD), isolated from an 80-yr-old patient with stage 3B nonsmall cell lung cancer, was cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine (all from Gibco Invitrogen, Carlsbad, CA). Experiments were performed with 70–100% confluent cells from passages 49–55. Before the experiments, cells were grown in reduced-serum (RPMI + 1% FBS) for 16 h. All cells were maintained in 5% CO<sub>2</sub> and 95% room air in a water-jacketed 37°C incubator.

### Experimental Procedure

Cell monolayers were incubated either with *Escherichia coli* serotype 055:B5 lipopolysaccharide (Sigma-Aldrich) at a concentration of 4  $\mu$ g/ml diluted in RPMI + 1% FBS medium for 4 h, or with TNF- $\alpha$  (Gibco Invitrogen) at a concentration of 20 ng/ml, also diluted in RPMI-1% FBS, for 20 min for Western blot analysis or 4 h for assessment of cell migration and cytotoxic effects.

Ropivacaine 0.5% (Naropin®, APP Pharmaceuticals, Schaumburg, IL), lidocaine 2% (APP Pharmaceuticals), or chloroprocaine 3% (Bedford Laboratories, Bedford, OH) were diluted with RPMI + 1% FBS medium to achieve the concentrations tested (1 nM–100  $\mu$ M) for the treatment of H838 cells in presence or absence of TNF- $\alpha$  or lipopolysaccharide. For some experiments, cells were pretreated with the VGSC agonist veratridine (Sigma-Aldrich) for 30 min or with the VGSC antagonist tetrodotoxin (Sigma-Aldrich) for 10 min.

### Cytotoxicity Assay

Cytotoxicity was measured using the Cytotoxicity Detection KitPlus (Roche, Indianapolis, IN), following the instructions by the manufacturer. The assay measures the activity of lactate dehydrogenase as a marker for cytotoxicity in cell culture supernatants. NCI-H838 cells were incubated for 4 h in low serum RPMI-1640 medium (1% FBS, 1% penicillin/streptomycin, 1% L-glutamine) with different concentrations of ropivacaine, lidocaine, or chloroprocaine (1 nM–100  $\mu$ M) in presence or absence of TNF- $\alpha$  at a concentration of 20 ng/ml. Thirty min before the end of the experiment, some cells were lysed by the addition of 10% Triton-X 100 (Sigma-Aldrich) to measure the maximal release of lactate dehydrogenase. Then the supernatant was collected and centrifuged for 5 min at 700 g to remove all cellular debris. Lactate dehydrogenase content was determined by the measurement of red formazan, derived from the yellow tetrazolium salt INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) by a catalyst after reduction of NAD<sup>+</sup> to NADH + H<sup>+</sup> by lactate dehydrogenase. Cytotoxicity was then calculated according to the following formula:

$$\text{Cytotoxicity \%} = \frac{(\text{experimental release} - \text{background})}{(\text{maximum release} - \text{background})} \times 100$$

### Cell Harvest and Lysis

After the end of the experiment, cells were washed once with ice-cold Dulbecco's phosphate-buffered saline (Cellgro, Manassas, VA) and lysed with radioimmunoprecipitation buffer (Boston Bioproducts, Ashland, MA) supplemented with protease inhibitor cocktail, 200 mM phenylmethylsulfonyl-fluoride, 1 mM EDTA, 1 mM sodium-fluoride, and 1 mM sodium-orthovanadate (all from Sigma-Aldrich). After a brief sonification and 10 min centrifugation at 4°C and 13,200 rpm in an Eppendorf 5415R microcentrifuge (Eppendorf AG, Hamburg, Germany), the supernatant was collected and stored at -20°C until further use. Total protein concentration was determined using a BCA protein assay kit (Pierce Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions, using albumin to generate a standard curve.

### Western Blot Analysis

Before boiling the lysates for 5 min, 6× Laemmli sample buffer (Boston Bioproducts) was added at a final concentration of 1× as well as 30 mM Dithiothreitol (DTT, Biorad, Hercules, CA). Equal amounts of protein (10 µg) were loaded in each lane of a 10% polyacrylamide gel for sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Biorad) with 5% blotting grade nonfat dry milk (Biorad) dissolved in Tris-buffered saline with 0.05% TWEEN-20 (TBST) (Sigma-Aldrich) for 30 min. The membranes were then probed with primary antibodies in TBST for 2 h on a shaker at room temperature or at 4°C overnight. Three washes with TBST were followed by incubation of the membrane with horseradish-peroxidase-conjugated secondary antibody (KPL, Gaithersburg, MD) in 5% milk in TBST for 1 h on a shaker at room temperature. After four washes with TBST, enhanced chemiluminescence solution (Thermo Scientific) was used to visualize the bands on HyBlot CL film (Denville, South Plainfield, NJ). To detect the total amount of ICAM-1 or Src on the same membranes after detection of the phosphorylated forms of the proteins, the antibodies were removed from the membranes using Restore Western Blot Stripping Buffer (Thermo Scientific). Subsequently, the membranes were blocked and reprobed with primary and secondary antibodies, as stated above.

Primary antibodies for pY419 Src and (total) Src were purchased from Cell Signaling Technologies (Danvers, MA) and for pY512 ICAM-1 and (total) ICAM-1 from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell Migration Assay

NCI-H838 lung cancer cells were suspended at a concentration of 10<sup>6</sup>/ml and were stained with fluorescent dye (Cytoselect®, Cell Biolabs, San Diego, CA). Then, 10<sup>5</sup> NCI-H838 cells were

resuspended in 300 µl RPMI-1640 medium with 0% FBS and no other supplements or with different concentrations of ropivacaine, lidocaine, or chloroprocaine (1 pM–100 µM) and were incubated for 15 min at room temperature and placed into the upper chamber of a 6.5-mm Transwell® polycarbonate membrane insert with 8-µm pores (Corning Life Sciences, Lowell, MA). The inserts were then placed into 500 µl complete medium (RPMI-1640, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, plus the same concentration of local anesthetic as present in the upper chamber) in a 24-well plate. To assess the reversibility of the inhibition of cell migration, cells were washed three times after incubation with ropivacaine before resuspending in 300 µl RPMI-1640 medium and placement into the upper chamber of a Transwell® insert. After 4 h, all inserts were removed from the well. The remaining fluid in the upper chamber was removed and the inserts were placed into fresh wells filled with cell detachment and lysis solution. A Wallac Victor2® microplate reader (Perkin Elmer, Waltham, MA) was used to detect fluorescence at 485/535 nm (excitation/emission).

### Statistical Analysis

Normal distribution of all data were first assessed with a Shapiro–Wilk test. Normally distributed data (cytotoxicity) are presented as column scatter plots with the mean of each group indicated by a horizontal line. These results were analyzed using three-way ANOVA with the local anesthetics, their concentration, and the presence or absence of TNF-α as factors to be tested. All other data (densitometry, migration), also presented as column scatter plots with a horizontal line indicating the mean, were analyzed using nonparametric testing methods (Kruskal–Wallis and Mann–Whitney U test). In order to maintain the family-wise error rate below 0.05, the result of multiple comparisons (Mann–Whitney U tests) following a statistically significant Kruskal–Wallis test were evaluated for significance by the Simes–Hochberg method. All tests were performed in a nonblinded and two-tailed manner with the SPSS Statistics Software Version 19 (IBM, Zurich, Switzerland). Graphs were generated using GraphPad Prism for Mac, Version 5 (GraphPad Software, La Jolla, CA). All experiments were performed at least three times. *P* < 0.05 was considered statistically significant.

## Results

### Effect of Ropivacaine, Lidocaine, and Chloroprocaine on Cytotoxicity

First the cytotoxic effect of different local anesthetics was evaluated by measuring the activity of lactate dehydrogenase in cell culture supernatants after incubation of NCI-H838 lung cancer cells with different concentrations (1 nM, 1 µM, 10 µM, 100 µM) of ropivacaine, lidocaine, and chloroprocaine in the absence or presence of TNF-α at a concentration of 20 ng/ml for 4 h. Untreated cells served as control. The overall data analysis was conducted using three-way



ANOVA. Cytotoxicity was the dependent factor and the type of local anesthetic, its concentration, and the presence or absence of TNF- $\alpha$  were the factors to be investigated. The analysis revealed that only the presence of TNF- $\alpha$  significantly increased cytotoxicity ( $P = <0.001$ ). Neither the application of the different local anesthetics ( $P = 0.264$ ) nor their different concentrations ( $P = 0.919$ ) had any influence on cytotoxicity measures. An analysis of the interaction of the local anesthetics with their different concentrations ( $P = 0.908$ ) or with TNF- $\alpha$  ( $P = 0.358$ ) did not reach statistical significance. The same result could be observed for the combined analysis of TNF- $\alpha$  together with the different concentrations of the local anesthetics ( $P = 0.979$ ) or a combination of all three factors ( $P = 0.992$ ).

#### Effect of Ropivacaine on the Phosphorylation Status of Src and ICAM-1 in NCI-H838 Lung Cancer Cells

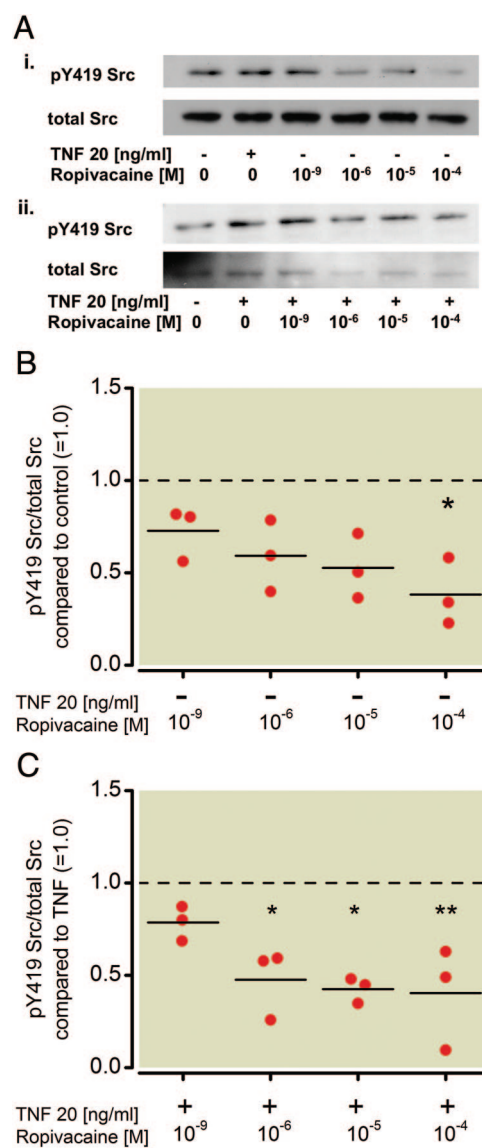
NCI-838 lung cancer cells were first incubated for 20 min with TNF- $\alpha$  at a concentration of 20 ng/ml in the presence or absence of different concentrations of ropivacaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M). Whole cell lysates were analyzed by Western blot analysis, probing for Src, phosphorylated at tyrosine 419 (pY419 Src) and total Src (fig. 1A).

Incubation of cells with ropivacaine alone for 20 min led to a significant decrease in phosphorylation of Src compared with untreated cells (= control) of 62% by 100  $\mu$ M ( $P = 0.003$ ; fig. 1B). Ten  $\mu$ M ropivacaine led to a 48% decrease, which was not considered statistically significant ( $P = 0.022$ , fig. 1B). Coincubation of TNF- $\alpha$  with different concentrations of ropivacaine showed that ropivacaine also decreased the phosphorylation of Src at tyrosine 419 in a significant manner compared with TNF- $\alpha$ , alone even at 1  $\mu$ M, by 52% ( $P = 0.022$ , fig. 1C). Higher concentrations of ropivacaine led to an even greater attenuation of TNF- $\alpha$ -induced Src phosphorylation (58% by 10  $\mu$ M,  $P = 0.006$ , and 60% by 100  $\mu$ M,  $P = 0.017$ ; fig. 1C).

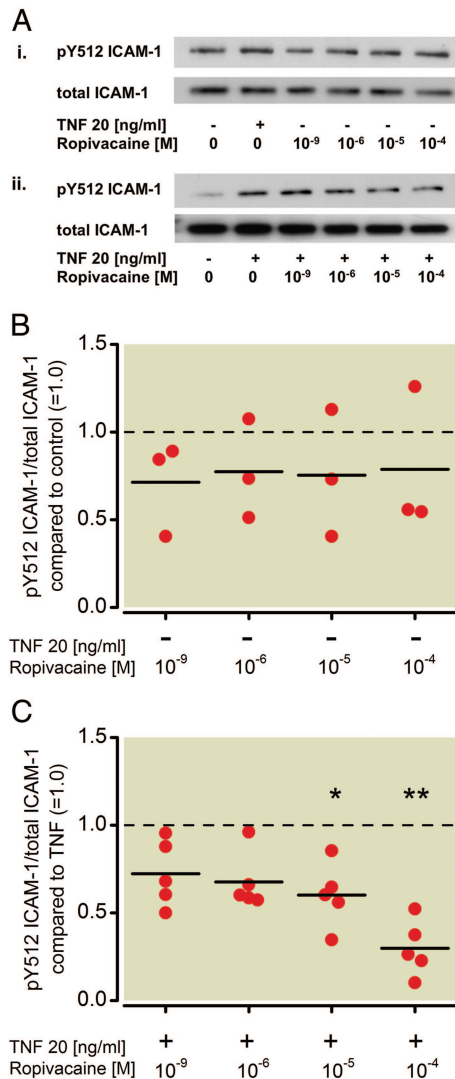
The same whole cell lysates were also analyzed *via* Western blot probing for ICAM-1, phosphorylated at tyrosine 512 (pY512 ICAM-1) and total ICAM-1 (fig. 2A). Increasing concentrations of ropivacaine did not alter the phosphorylation status of ICAM-1 (Kruskal–Wallis test,  $P = 0.99$ ; fig. 2B), but coincubation with ropivacaine resulted in a significant attenuation of TNF- $\alpha$ -induced phosphorylation of ICAM-1 at tyrosine 512 compared with treatment with TNF- $\alpha$  alone, with significant inhibition beginning at 10  $\mu$ M (40% decrease,  $P = 0.01$ ; fig. 2C). A higher dose of ropivacaine further decreased TNF- $\alpha$ -induced ICAM-1 phosphorylation (71% by 100  $\mu$ M,  $P < 0.001$ ; fig. 2C). A 32% decrease by 1  $\mu$ M was also observed but not considered statistically significant ( $P = 0.031$ , fig. 2C).

#### Effect of Lidocaine on the Phosphorylation Status of Src and ICAM-1 in NCI-H838 Lung Cancer Cells

To evaluate the effect of lidocaine on NCI-H838 lung cancer cell Src signaling, cells were treated with increasing concen-



**Fig. 1.** Effect of ropivacaine on the phosphorylation status of Src in NCI-H838 lung cancer cells. (A) (i) Representative Western blot of NCI-H838 cell Src, phosphorylated at tyrosine 419 (pY419 Src, row 1) and total Src (row 2) after treatment with either tumor necrosis factor (TNF)- $\alpha$  (20 ng/ml) or with different concentrations of ropivacaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (ii) Representative blot of NCI-H838 lung cancer cell pY419 Src (row 1) and total Src (row 2) after treatment with TNF- $\alpha$  (20 ng/ml) with or without different concentrations of ropivacaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (B) Relative density of the Western blot bands of pY419 Src normalized to the densitometry values of total Src compared with control (= 1.0, dashed line) in the absence of TNF- $\alpha$  expressed. Data from three independent experiments shown as scatter plot ( $n = 3$ ). Horizontal line indicates mean for each group; \* $P < 0.05$  versus control. (C) Relative density of the Western blot bands of pY419 Src normalized to the densitometry values of total Src after coincubation of ropivacaine with TNF- $\alpha$  (20 ng/ml) compared with TNF- $\alpha$  alone (= 1.0, dashed line). Data from three independent experiments shown as scatter plot ( $n = 3$ ). Horizontal line indicates mean for each group; \* $P < 0.05$  versus TNF- $\alpha$ , \*\* $P < 0.01$  versus TNF- $\alpha$ .



**Fig. 2.** Effect of ropivacaine on the phosphorylation status of intercellular adhesion molecule-1 (ICAM-1) in NCI-H838 lung cancer cells. (A) (i) Representative Western blot of NCI-H838 lung cancer cell ICAM-1, phosphorylated at tyrosine 512 (pY512 ICAM-1, row 1) and total ICAM-1 (row 2) after treatment with either tumor necrosis factor (TNF)- $\alpha$  (20 ng/ml) or different concentrations of ropivacaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (ii) Representative blot of NCI-H838 cell pY512 ICAM-1 (row 1) and total ICAM-1 (row 2) after treatment with TNF- $\alpha$  (20 ng/ml) in the presence or absence of different concentrations of ropivacaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (B) Relative density of the Western blot bands of pY512 ICAM-1 normalized to the densitometry values of total ICAM-1 compared with control (= 1.0, dashed line) in the absence of TNF- $\alpha$ . Data from three independent experiments shown as scatter plot ( $n = 3$ ). Horizontal line indicates mean for each group. (C) Relative density of the Western blot bands of pY512 ICAM-1 normalized to the densitometry values of total ICAM-1 after co-incubation of ropivacaine with TNF- $\alpha$  (20 ng/ml) compared with TNF- $\alpha$  alone (= 1.0, dashed line). Data from five independent experiments shown as scatter plot ( $n = 5$ ). Horizontal line indicates mean for each group; \* $P < 0.05$  versus TNF- $\alpha$ , \*\* $P < 0.01$  versus TNF- $\alpha$ . ICAM-1 = intercellular adhesion molecule-1.

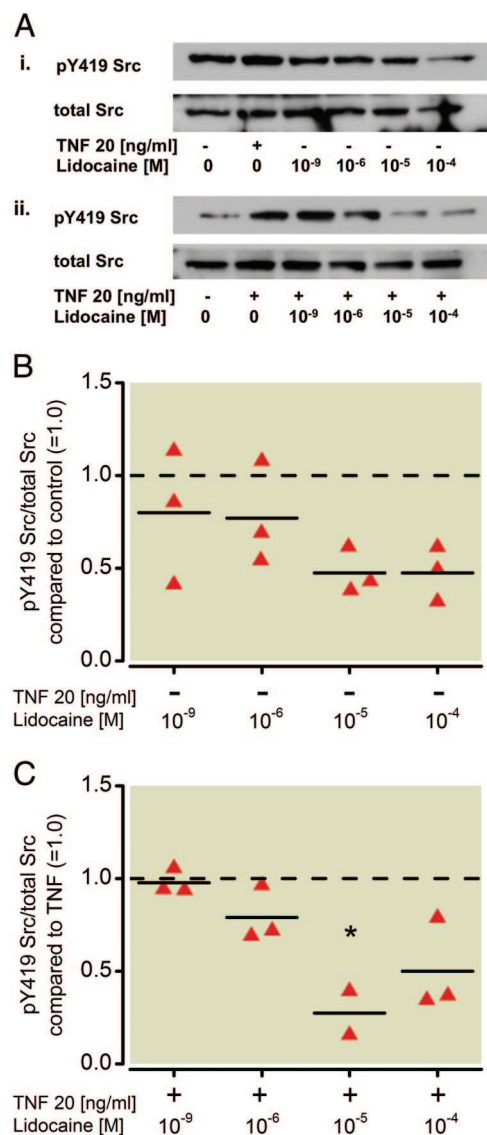
treatments of lidocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min and again analyzed for Src phosphorylation *via* Western blot (fig. 3A). Although a dose-dependent decrease in Src phosphorylation at tyrosine 419 was observed after incubation of the cells with lidocaine for 20 min, this decrease did not reach statistical significance (Kruskal–Wallis test,  $P = 0.146$ ; fig. 3B). However, a significant decrease in TNF- $\alpha$ -induced Src phosphorylation of 73% was observed after co-incubation of cells with TNF- $\alpha$  and 10  $\mu$ M ( $P = 0.012$ ) of lidocaine (fig. 3C). ICAM-1 phosphorylation in the Western blot (fig. 4A) was not affected by lidocaine alone (Kruskal–Wallis test,  $P = 0.624$ ; fig. 4B), but when co-incubated with TNF- $\alpha$ , lidocaine reduced ICAM-1 phosphorylation compared with TNF- $\alpha$  alone in a significant manner (73% decrease by 100  $\mu$ M,  $P = 0.002$ ; fig. 4C). An attenuation of 50% was observed after co-incubation of 1  $\mu$ M lidocaine with TNF- $\alpha$ , but did not reach statistical significance ( $P = 0.022$ ; fig. 4C).

#### Effect of Chlorprocaine on the Phosphorylation Status of Src and ICAM-1 in NCI-H838 Lung Cancer Cells

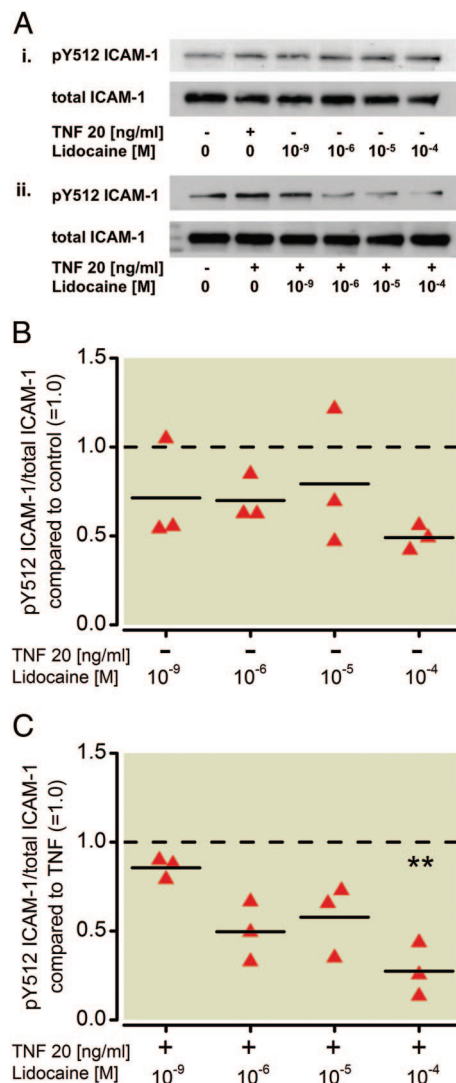
To compare the results observed with the amide-linked local anesthetics (ropivacaine, lidocaine) with an ester-linked local anesthetic, NCI-H838 lung cancer cells were treated as above with increasing concentrations of the ester-linked chlorprocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M). Analysis of Src phosphorylation (fig. 5A) in H838 cell lysates showed that chlorprocaine had no effect on Src activation, either alone (Kruskal–Wallis test,  $P = 0.305$ ; fig. 5B) or when co-incubated with TNF- $\alpha$  compared with TNF- $\alpha$  alone ( $P = 0.443$ ; fig. 5C). There was also no effect of chlorprocaine on ICAM-1 phosphorylation (fig. 6A) after incubation with chlorprocaine alone compared with control (Kruskal–Wallis test,  $P = 0.924$ ; fig. 6B), and after co-incubation with TNF- $\alpha$  ( $P = 0.217$ ; fig. 6C).

#### Effect of Sodium Channel Activator Veratridine and Sodium-channel Blocker Tetrodotoxin on Phosphorylation Status of Src and ICAM-1 in NCI-H838 Lung Cancer Cells

To investigate whether the VGSC plays a role in activation of Src and subsequent phosphorylation of ICAM-1, NCI-H838 lung cancer cells were treated with the alkaloid and VGSC agonist veratridine at a concentration of 0.015 mM for 45 min before the cells were harvested, lysed, and prepared for Western blot analysis of pY419 Src and pY512 ICAM-1, as well as the total amounts of each protein (fig. 7Ai and ii). This concentration was chosen because it was shown that 7.4  $\mu$ M veratridine abolishes lidocaine-induced membrane depolarization of intestinal cells.<sup>22</sup> For our experiments, we doubled this concentration. Densitometry analysis revealed that neither Src activation nor ICAM-1 phosphorylation was affected by treatment with veratridine compared with control (Kruskal–Wallis test,  $P = 0.98$ ; fig. 7Aiii).

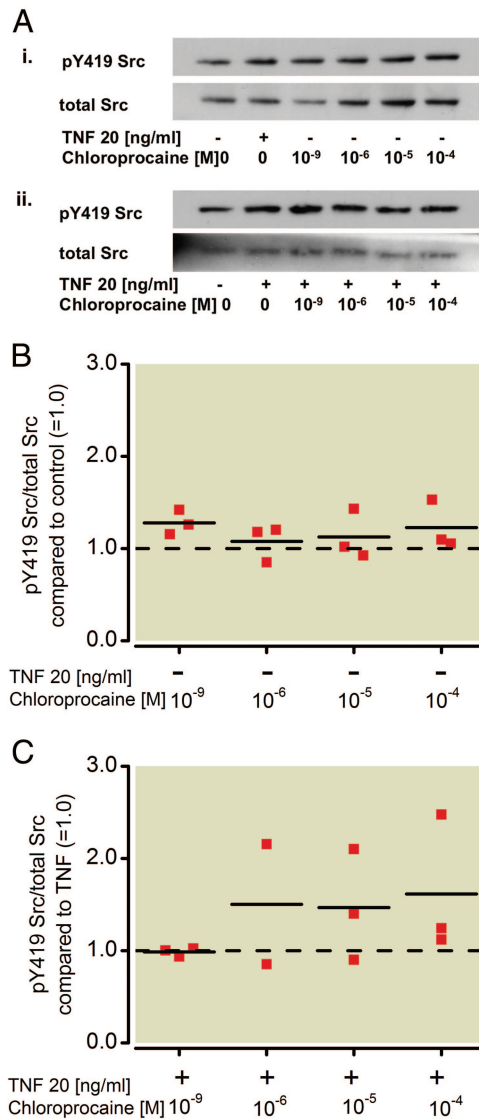


**Fig. 3.** Effect of lidocaine on the phosphorylation status of Src in NCI-H838 lung cancer cells. (A) (i) Representative Western blot of NCI-H838 cell Src, phosphorylated at tyrosine 419 (pY419 Src, row 1) and total Src (row 2) after treatment with either tumor necrosis factor (TNF)- $\alpha$  (20 ng/ml) or with different concentrations of lidocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (ii) Representative blot of NCI-H838 lung cancer cell pY419 Src (row 1) and total Src (row 2) after treatment with TNF- $\alpha$  (20 ng/ml) with or without different concentrations of lidocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (B) Relative density of the Western blot bands of pY419 Src normalized to the densitometry values of total Src compared with control (= 1.0, dashed line) in the absence of TNF- $\alpha$ . Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group. (C) Relative density of the Western blot bands of pY419 Src normalized to the densitometry values of total Src after coincubation of lidocaine with TNF- $\alpha$  (20 ng/ml) compared with TNF- $\alpha$  alone (= 1.0, dashed line). Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group; \* $P$  < 0.05 versus TNF- $\alpha$ .

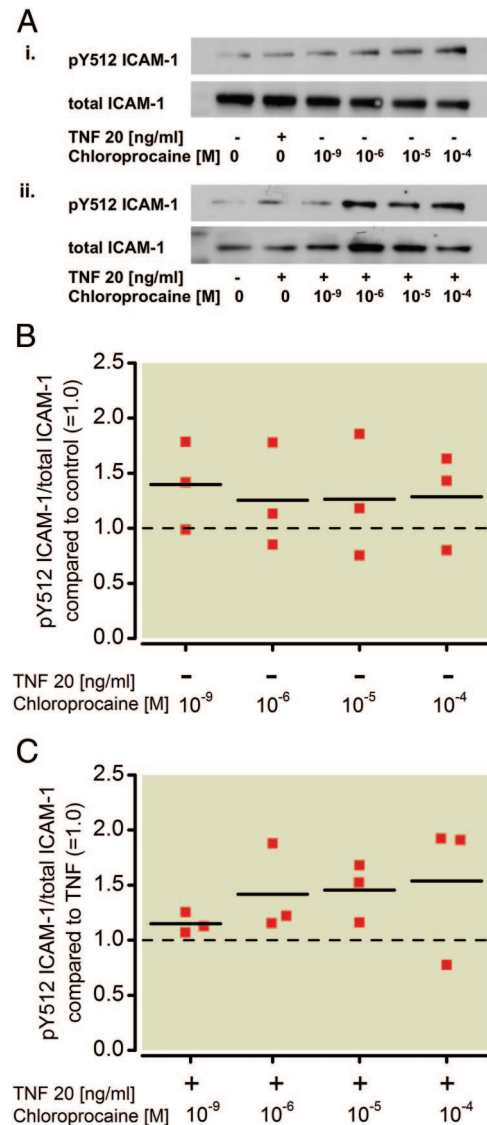


**Fig. 4.** Effect of lidocaine on the phosphorylation status of intercellular adhesion molecule-1 (ICAM-1) in NCI-H838 lung cancer cells. (A) (i) Representative Western blot of NCI-H838 lung cancer cell ICAM-1, phosphorylated at tyrosine 512 (pY512 ICAM-1, row 1) and total ICAM-1 (row 2) after treatment with either tumor necrosis factor (TNF)- $\alpha$  (20 ng/ml) or different concentrations of lidocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (ii) Representative blot of NCI-H838 cell pY512 ICAM-1 (row 1) and total ICAM-1 (row 2) after treatment with TNF- $\alpha$  (20 ng/ml) in the presence or absence of different concentrations of lidocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (B) Relative density of the Western blot bands of pY512 ICAM-1 normalized to the densitometry values of total ICAM-1 compared with control (= 1.0, dashed line) in the absence of TNF- $\alpha$ . Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group. (C) Relative density of the Western blot bands of pY512 ICAM-1 normalized to the densitometry values of total ICAM-1 after coincubation of lidocaine with TNF- $\alpha$  (20 ng/ml) compared with TNF- $\alpha$  alone (= 1.0, dashed line). Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group; \*\* $P$  < 0.01 versus TNF- $\alpha$ . ICAM-1 = intercellular adhesion molecule-1.



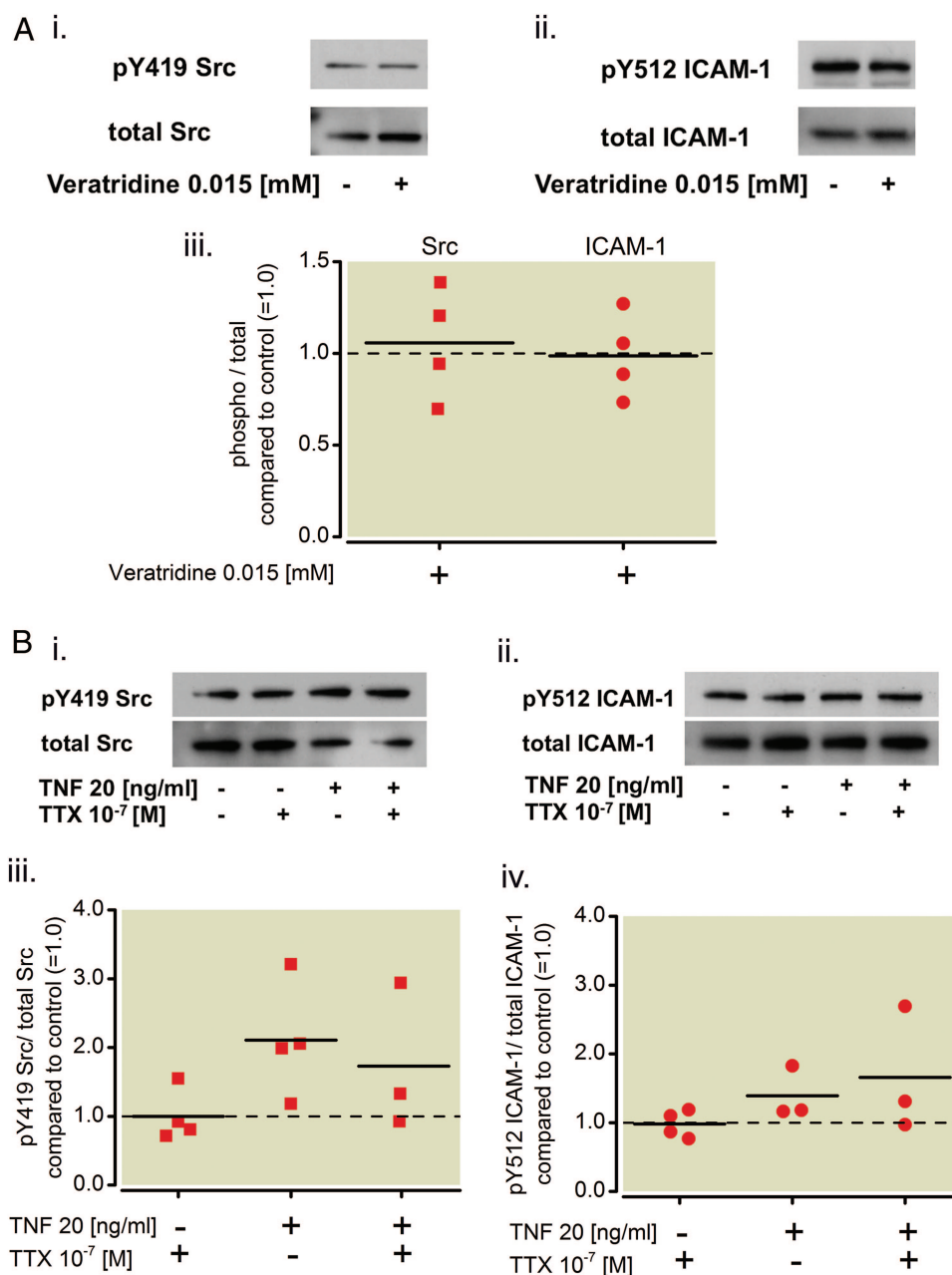


**Fig. 5.** Effect of chlorprocaine on the phosphorylation status of Src in NCI-H838 lung cancer cells. (A) (i) Representative Western blot of NCI-H838 cell Src, phosphorylated at tyrosine 419 (pY419 Src, row 1) and total Src (row 2) after treatment with either tumor necrosis factor (TNF)- $\alpha$  (20 ng/ml) or with different concentrations of chlorprocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (ii) Representative blot of NCI-H838 lung cancer cell pY419 Src (row 1) and total Src (row 2) after treatment with TNF- $\alpha$  (20 ng/ml) with or without different concentrations of chlorprocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (B) Relative density of the Western blot bands of pY419 Src normalized to the densitometry values of total Src compared with control (= 1.0, dashed line) in the absence of TNF- $\alpha$ . Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group. (C) Relative density of the Western blot bands of pY419 Src normalized to the densitometry values of total Src after coincubation of chlorprocaine with TNF- $\alpha$  (20 ng/ml) compared with TNF- $\alpha$  alone (= 1.0, dashed line). Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group.

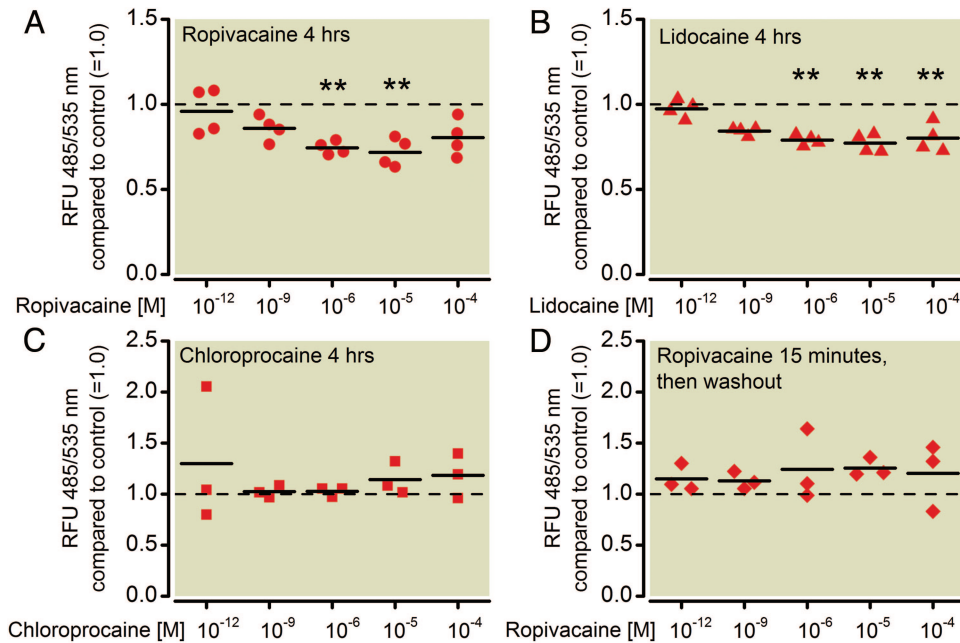


**Fig. 6.** Effect of chlorprocaine on the phosphorylation status of intercellular adhesion molecule-1 (ICAM-1) in NCI-H838 lung cancer cells. (A) (i) Representative Western blot of NCI-H838 lung cancer cell ICAM-1, phosphorylated at tyrosine 512 (pY512 ICAM-1, row 1) and total ICAM-1 (row 2) after treatment with either tumor necrosis factor (TNF)- $\alpha$  (20 ng/ml) or different concentrations of chlorprocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (ii) Representative blot of NCI-H838 cell pY512 ICAM-1 (row 1) and total ICAM-1 (row 2) after treatment with TNF- $\alpha$  (20 ng/ml) in the presence or absence of different concentrations of chlorprocaine (1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 20 min. (B) Relative density of the Western blot bands of pY512 ICAM-1 normalized to the densitometry values of total ICAM-1 compared with control (= 1.0, dashed line) in the absence of TNF- $\alpha$ . Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group. (C) Relative density of the Western blot bands of pY512 ICAM-1 normalized to the densitometry values of total ICAM-1 after coincubation of lidocaine with TNF- $\alpha$  (20 ng/ml) compared with TNF- $\alpha$  alone (= 1.0, dashed line). Data from three independent experiments shown as scatter plot (n = 3). Horizontal line indicates mean for each group.





**Fig. 7.** Effect of a sodium channel activator and blocker on the phosphorylation status of Src and ICAM-1 in NCI-H838 lung cancer cells. (A) (i) Representative Western blot of NCI-H838 cell Src, phosphorylated at tyrosine 419 (pY419 Src, row 1) and total Src (row 2) after treatment with veratridine (0.015 mM) for 45 min. (ii) Representative blot of NCI-H838 lung cancer cell pY512 ICAM-1 (row 1) and total Src (row 2) after treatment with veratridine (0.015 mM) for 45 min. (iii) Relative density of the Western blot bands of pY419 Src normalized to total Src (squares) and pY512 ICAM-1 normalized to total intercellular adhesion molecule-1 (ICAM-1) (circles) compared with control (= 1.0, dashed line) after treatment with veratridine (0.015 mM) for 45 min. Data from four independent experiments shown as scatter plot ( $n = 4$ ). Horizontal line indicates mean for each group. (B) (i) Representative Western blot of NCI-H838 cell Src, phosphorylated at tyrosine 419 (pY419 Src, row 1) and total Src (row 2) after pretreatment with tetrodotoxin (100 nM) for 10 min and subsequent addition of tumor necrosis factor (TNF)- $\alpha$  (20 ng/ml) for 20 min. (ii) Representative Western blot of NCI-H838 cell ICAM-1, phosphorylated at tyrosine 512 (pY512 ICAM-1, row 1) and total ICAM-1 (row 2) after pretreatment with TTX (100 nM) for 10 min and subsequent addition of TNF- $\alpha$  (20 ng/ml) for 20 min. (iii) Relative density of the Western blot bands of pY419 Src normalized to the densitometry values of total Src compared with control (= 1.0, dashed line) after pretreatment with tetrodotoxin (100 nM) for 10 min and subsequent addition of TNF- $\alpha$  (20 ng/ml) for 20 min. Data from four independent experiments shown as scatter plot ( $n = 4$ ). Horizontal line indicates mean for each group. (iv) Relative density of the Western blot bands of pY512 ICAM-1 normalized to the densitometry values of total ICAM-1 after pretreatment with tetrodotoxin (100 nM) for 10 min and subsequent addition of TNF- $\alpha$  (20 ng/ml) for 20 min compared with control (= 1.0, dashed line). Data from three independent experiments shown as scatter plot ( $n = 3$ ). Horizontal line indicates mean for each group. ICAM-1 = intercellular adhesion molecule-1; TTX = tetrodotoxin.



**Fig. 8.** Migratory ability of NCI-H838 lung cancer cells in the presence of ropivacaine, lidocaine, and chloroprocaine. Determination of *in vitro* transmigration of NCI-H838 cells through a polycarbonate membrane after 4 h in the absence or presence of different concentrations (1 pM, 1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) of (A) ropivacaine, (B) lidocaine, and (C) chloroprocaine. (D) Determination of *in vitro* transmigration of NCI-H838 cells through a polycarbonate membrane after 4 h in the absence or presence of different concentrations (1 pM, 1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) of ropivacaine for only 15 min followed by a wash of the cells with fresh medium right before the start of the assay. Cells were stained with a fluorescent dye before the start of the assay and lysed after migration through the membrane. Fluorescence was measured at 435/535 nm (excitation/emission). Values for untreated cells have been set as 1.0 (dashed line). Data from four (A and B) or three (C and D) independent experiments shown as scatter plot. Horizontal line indicates mean for each group; \*\* $P < 0.01$  versus control. RFU = relative fluorescence units.

The effect of tetrodotoxin, a nonlocal anesthetic VGSC antagonist, was investigated. Tetrodotoxin at a concentration of 100 nM ( $10^{-7}$  M) was used, as this concentration is known to inhibit all VGSCs in excitable membranes.<sup>23</sup> NCI-H838 lung cancer cells were pretreated with tetrodotoxin for 10 min before TNF- $\alpha$  (20 ng/ml) was added. Cells were lysed after 20 min. Representative Western blots of phosphorylated Src and ICAM-1 as well as the total amount of the proteins are shown in figure 7Bi and ii. Densitometry analysis revealed no difference in Src and ICAM-1 phosphorylation after pretreatment with tetrodotoxin and subsequent addition of TNF- $\alpha$  compared with TNF- $\alpha$  alone (Mann-Whitney U-test,  $P = 0.48$  for Src and  $P = 0.827$  for ICAM-1; fig. 7Biii and Biv).

#### Migratory Ability of NCI-H838 Lung Cancer Cells in the Presence of Ropivacaine, Lidocaine, and Chloroprocaine

NCI-H838 cells, stained with a fluorescent dye, were allowed to migrate through an 8- $\mu$ M-pore polycarbonate membrane for 4 h in the presence or absence of different concentrations of ropivacaine, lidocaine, or chloroprocaine (1 pM, 1 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M). Migrated cells were detached from the bottom of the membrane and lysed. Fluorescence was measured at 485/535 nm (excitation/emission) (fig. 8).

Compared with untreated cells (= control), ropivacaine induced a significant decrease in fluorescence – and therefore the number of cells migrated – by 26% ( $P = 0.005$ ) at 1  $\mu$ M concentration. A higher concentration decreased fluorescence by 28% (10  $\mu$ M,  $P = 0.003$ ; fig. 8A). This effect was completely abolished by removal of ropivacaine after the initial 15 min of incubation (Kruskal–Wallis test,  $P = 0.447$ ; fig. 8D).

Treatment with lidocaine decreased fluorescence after 4 h of migration (Kruskal–Wallis test,  $P = 0.004$ ). 1  $\mu$ M of lidocaine attenuated migration by 21% compared with control ( $P = 0.004$ ), whereas 10  $\mu$ M and 100  $\mu$ M led to a decrease of 23% ( $P = 0.002$ ) and 20% ( $P = 0.009$ ), respectively (fig. 8B). In contrast, incubation with different concentrations of chloroprocaine had no effect on fluorescence measured after 4 h (Kruskal–Wallis test,  $P = 0.718$ ; fig. 8C).

#### Discussion

The results of this study indicate that the amide-linked local anesthetics ropivacaine and lidocaine inhibit Src (auto)phosphorylation at tyrosine 419 induced by inflammatory stimuli such as TNF- $\alpha$ , and that ropivacaine inhibited Src phosphorylation (activity) even in the absence of inflammatory stimuli. In contrast, the ester-linked local anesthetic chloropro-

caine had no such effect. In addition, we observed decreased Src-mediated phosphorylation of ICAM-1 at tyrosine 512 in presence of ropivacaine and lidocaine in NCI-H838 lung cancer cells treated with TNF- $\alpha$  (and lipopolysaccharide). Moreover, ropivacaine and lidocaine were shown to inhibit the migration of H838 cancer cells.

Interestingly, the inhibitory effect on either the phosphorylation of Src and ICAM-1 or migration of lung cancer cells was observed with amide local anesthetics (ropivacaine and lidocaine), but not with ester local anesthetics (chloroprocaine). Previous studies also demonstrated a selective protective effect of amide local anesthetics as compared with the ester-linked compounds. De Klaver *et al.* demonstrated that lidocaine, ropivacaine, and bupivacaine protected endothelial cells against lipopolysaccharide-induced cellular injury, whereas procaine and tetracaine did not.<sup>24</sup>

Recently, Src tyrosine protein kinase has become a key research focus of cancer development, growth, and metastasis. Src is involved in signaling epithelial-to-mesenchymal transformation, *e.g.*, *via* loss of E-cadherin and subsequently decreased cell-to-cell adhesion<sup>16,17,25</sup> necessary for solid tumor metastasis.<sup>18</sup> Src is activated either by (auto)phosphorylation at tyrosine 419 and/or by dephosphorylation at tyrosine 529.<sup>26</sup> This study showed that ropivacaine dose-dependently decreased Src activation in NCI-H838 lung cancer cells, even in the absence of an inflammatory stimulus (TNF- $\alpha$  or lipopolysaccharide), although stimulation was necessary to observe an effect of ropivacaine on Src-dependent ICAM-1 phosphorylation at tyrosine 512. This indicates that Src might be the primary target of ropivacaine and that phosphorylation of ICAM-1 is attenuated secondary to the inhibition of Src activity, as ICAM-1 was previously shown to be a Src substrate.<sup>15</sup>

Local anesthetics are known to block the VGSC.<sup>27</sup> The inhibitory effect of ropivacaine and lidocaine on Src activation and ICAM-1 phosphorylation, as observed in this study, is independent from the inhibition of the VGSC. No change in the phosphorylation status of the two proteins after incubation with the sodium channel agonist veratridine was observed. Neither Src nor ICAM-1 phosphorylation induced by TNF- $\alpha$  was altered by pretreatment with the sodium channel inhibitor tetrodotoxin. These results are in accordance with those of another study that demonstrated VGSC-independent inhibition of TNF- $\alpha$ -induced epithelial chemokine secretion.<sup>28</sup> However, there is some evidence that VGSCs linked with Fyn, another member of the Src protein tyrosine kinase family, might play a role in the migration of neuronal cells and metastasis.<sup>29</sup> Also, an inhibition of VGSCs in other human nonsmall-cell lung cancer cell lines led to a reduction of *in vitro* invasion of these malignant cells.<sup>30</sup>

The identification of Src as a key enzyme in tumor growth and metastasis has led to the development of several "targeted therapies," such as Src-inhibitors or combined Bcr/Abl and Src-inhibitors (*e.g.*, Dasatinib®, Bristol-Myers Squibb, New York, NY).<sup>31</sup> Most of these compounds act as adenosine triphosphate-competitive inhibitors at the adenosine triphosphate-binding pocket of Src.<sup>32</sup>

It would therefore be interesting to determine in future studies how ropivacaine inhibits Src activity, assessing for example whether it competes with adenosine triphosphate for binding within the Src kinase domain.<sup>33</sup>

ICAM-1, a member of the immunoglobulin superfamily of genes that play key roles in the binding of leukocytes to the vascular endothelium, is also involved in growth and metastasis of cancer. TNF- $\alpha$  stimulation of NCI-H838 cells (those used in the current study) for 16 h increased ICAM-1 expression.<sup>12</sup> Increased expression of ICAM-1 could be linked to a more aggressive tumor phenotype<sup>34,35</sup> and to enhanced leukocyte infiltration and binding to tumor cells.<sup>36</sup> In addition, it was shown that A549 lung cancer cells overexpressing ICAM-1 exhibited enhanced *in vitro* cell migration and *in vivo* metastasis, an effect that could be inhibited by an anti-ICAM-1 antibody.<sup>35</sup>

This study focused on the phosphorylation of ICAM-1, which is necessary for rapid TNF- $\alpha$ -induced clustering of ICAM-1 resulting in enhanced neutrophil binding.<sup>15</sup> The fact that ropivacaine and lidocaine inhibited this phosphorylation might be beneficial in the setting of metastasis. They might also attenuate the binding of circulating cancer cells to the vascular endothelium and therefore reduce transmigration and metastasis.

The well-established cytotoxic effect of local anesthetics, *e.g.*, on neuronal cells,<sup>37</sup> is not considered in the interpretation of our results. The concentrations of ropivacaine, lidocaine, and chloroprocaine used in this study showed that they did not induce cytotoxic effects after 4 h of treatment. In addition, an alteration in TNF- $\alpha$ -induced cytotoxicity in our lung cancer cell line was not observed.

Another finding was the inhibition of tumor cell migration by ropivacaine and lidocaine at 1  $\mu$ M. Src is known to play a key role in cell migration, which is necessary for cancer cells to metastasize.<sup>18</sup> It regulates cytoskeletal changes required for cell migration by phosphorylating proteins associated with focal adhesions and actin bundling, which control cell membrane protrusions.<sup>38,39</sup> Src is also an upstream regulator of  $\rho$  family GTPases such as Rac and  $\rho$ , which together regulate dynamic changes in the cytoskeleton and control the disassembly of actin-based cytoskeletal structures and cell-matrix adhesions.<sup>17</sup> We therefore hypothesize that the inhibition of tumor cell migration by amide local anesthetics is because of the inhibition of Src. The fact that the observed effect of inhibition of migration could be abolished by wash-out of the local anesthetic after 15 min (as shown for ropivacaine) also indicates, first, that the observed effect is not because of a cytotoxic effect of the local anesthetic, and second, that this effect is reversible.

It is well known that hematogenous dissemination of cancer cells occurs during the surgical resection of a tumor.<sup>40</sup> Circulating tumor cells, detected in the blood 24 h postoperatively, are an independent prognostic marker for cancer recurrence because of the ability of these cells to extravasate and metastasize.<sup>41</sup> In accordance with the results of our

study, we suggest that the perioperative administration of local anesthetics may have the potential added benefit of attenuating extravasation and metastasis of circulating tumor cells before initiation of systemic treatment with cytotoxic agents.

Thus far, the potential beneficial effect of regional anesthesia to improve long-term outcome after cancer surgery has been attributed to inhibition of the surgical stress response and to the decrease in opioid requirements. It has been shown that opioids even at clinically useful concentrations might promote migration and proliferation of tumor cells, *e.g.*, in breast cancer.<sup>42,43</sup> This work reveals to the best of our knowledge a possible mechanism by which local anesthetics might be beneficial in patients with cancer and/or undergoing cancer surgery and that this mechanism may be because of, at least in part, inhibition of Src tyrosine kinase, a key enzyme in cancer growth and metastasis. Future studies will focus on establishing the molecular mechanism of Src inhibition and the influence of this inhibition on ICAM-1 phosphorylation and function with comparison with established Src inhibitors.

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